SEMI-ANNUAL REPORT 62-2-4
July - December 1961

BASIC STUDIES IN PERCUTANEOUS ABSORPTION

Contract No. DA 18-108-CML 6537
Lever Brothers Co.

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BASIC STUDIES IN PERCUTANEOUS ABSORPTION

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I. INTRODUCTION

With the completion of the first year of research on "Basic Studies in Percutaneous Absorption" significant progress has been made in relating certain changes in skin state with increased permeability. Methods have been developed which allow consistent measurements of water diffusion rate, and of penetration rates of ions and simple chemical agents through skin. Possible differences in composition of skin fat from normal and essential fatty acid-deficient rats are reported, employing gas-liquid and thin film chromatographic methods. Work is in progress on the characterization of epidermal proteins.

This report describes work on permeability of skin from animals treated in the following ways:

1) Made deficient in essential fatty acids.

2) Made deficient in vitamins, A, B_6, and C, and trace metal magnesium.

3) Fed diets with low, standard and high levels of protein.

4) Topically treated with organic solvents.

Permeability data obtained on these animals are compared with results from normal animals in an effort to relate structural changes with the ability of skin to serve as a barrier.

II. CHEMICAL AND BIOLOGICAL PROPERTIES OF SKIN FROM RATS ON A FAT-FREE DIET

In continuing our studies on the fundamental nature of the skin barrier, the influence of specific nutritional factors on skin morphology and permeability is being investigated. The present study is concerned with the role of essential fatty acids (EFA) in maintaining the structure and functional capacity of skin.

Since the description of the EFA-deficiency syndrome by Burr and Burr (1) the need for EFA has been demonstrated in widely different species, including man. Among the more prominent manifestations of EFA-deficiency are reduced growth, impaired reproductive capacity, and pronounced skin alterations (2,3,4, 5).

Some of the biological, physical and chemical properties of skin of rats maintained on a fat-free (EFA-deficient) diet, have been determined. Efforts have been made to obtain a coordinated picture of the role of EFA in maintaining the integrity of the "barrier layer" of the skin.

Special Materials and Apparatus

Male rats having an initial body weight of about 40-55 g., obtained either from Hemlock Hollow Farms or from Sprague-Dawley, Inc. (Madison, Wisc.) were employed. The experimental group was maintained on the "Fat-Free Test Diet" supplied by Nutritional Biochemicals Corp. (Cleveland, O.),

with diet and water available ad libitum. The control animals were supplied with the same regimen supplemented at a level of 40 g. per kg. of diet with a mixture of partially hydrogenated vegetable oils containing 15% linoleic acid.

The $Ni^{63}Cl_2$ used for autoradiographic studies was obtained from the Nucleonic Corp. of America, Brooklyn, N. Y., and had an activity of 3,800 dps/ml.

For interference microscopy, the American Optical Interference Microscope, with a polarized light source, was employed.

The apparatus for water diffusion rate measurements of excised skin was fashioned after that of Burch and Winsor (6). Its construction and use are described in detail in a previous communication (7).

The penetration vessels used in the skin permeability studies were made to order by Kontes Glass Co., Vineland, N. J. The polyethylene rings used for mounting the skins on the penetration vessels were prepared from 36 mm. pill vial snap caps, by punching out a 25 mm. diameter disk from the center of the caps.

The apparatus for electrical conductivity measurements of intact skin was constructed after the design of Levine (8) and Blank and Finesinger (9). It has the following components: a 6-volt battery power supply, voltmeter (0-1 volt in 0.02

volt divisions), ammeter (0-15 microamperes in 0.2 microampere divisions), helipot (for voltage adjustment), electrode terminals, and foot-switch. The electrodes consist of round plastic rods drilled at one end to a depth of 5 mm., each containing a circular zinc plate 1.0 cm. in diameter. Contact with the skin surface to be measured is made by a gel prepared by mixing glycerine (50 parts), water (44 parts), hot saturated $ZnSO_4$ solution (5 parts), and agar (1 part) (10). Measurements are normally made at 1.0 volt, but the potential is lowered in cases where current exceeds 15 microamperes per volt.

Procedures

Histologic: - For study by standard light microscopy, skin is fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Sections cut at 4 microns are stained with the following: Giemsa stain, Walbach's modification (11), Laidlaw's reticulum stain (12), or picro-Gomori trichrome stain (13). For study by interference microscopy, unstained frozen sections, cut at 6-8 microns on the Harris-International cryostat, are employed.

Histochemical: - For study of sulfhydryl localization, skin is fixed in 80% ethanol containing 2% trichloroacetic acid, embedded in paraffin, and sections cut at 4 microns are treated by the technique of Barrnett and Seligman (14). For demonstration of nickel, dimethylglyoxime is employed (7).

Autoradiographic: - For studies on the permeability of (radioactive) nickel chloride, the following solutions are used:

1) $Ni^{63}Cl_2$, 0.5%
2) $Ni^{63}Cl_2$, 0.5% plus $C_{12}H_{25}SO_4Na$, 0.5% (above critical micelle concentration)

The test solutions are applied by cotton swab to the clipped dorsal area of each animal intermittently for one hour. Immediately after treatment, the tissue is excised and frozen sections are prepared. The liquid emulsion autoradiographic method described previously (7) is used to trace the tagged Ni^{63} in the skin sections.

Chemical: - In preparation of epidermis for chemical analysis, animal skin is first epilated using "Zip" wax. The epilated skin is exposed to ammonia vapor for about 40-60 minutes, and the epidermis removed as a sheet (15). The stripped epidermis is stored 1 hour in vacuo over H_2SO_4 to remove ammonia, followed by washing in benzene, then in ether, and homogenization in acetone. The sediment obtained after centrifugation is desiccated in vacuo over Drierite.

For analysis of total sulfhydryl content, a portion of the dried epidermal powder is incubated in 3M aqueous urea solution for 1 hour at 40°C. (16). The tissue suspension is then reacted with an amyl acetate solution of 1-(4-chloromercuriphenylazo)-naphthol-2 according to the method of Flesch and Kun (17).

For determination of total protein sulfur as cystine, by the method of Sullivan et al. (18), the following procedure is employed (19): a portion of dried epidermal powder is weighed out, and two precipitations with 5% trichloroacetic acid carried out in the cold, followed by ethanol washing, two extractions with hot ethanol:ether (3:1), and desiccation in vacuo. After re-weighing, the material is hydrolyzed in the autoclave at a-bout 17 pounds pressure for 8 hours, with 3N HCl. The hydrolyzate is evaporated to dryness over NaOH pellets, diluted to a known volume with 0.1N HCl and filtered. Cystine content is estimated by reaction with sodium 1,2-naphthoquinone-4-sulfonate after cyanide reduction.

Penetration Rate Determination: - Rat skin permeability was studied by the manometric technique described in the last report (7). The general procedure employed in setting up the skin penetration vessels is as follows:

About 18 to 20 hours prior to sacrifice, the fur is clipped from the trunk area of rats. The animals are sacrificed by cervical fracture and the skin is dissected from the trunk area in one piece. The subcutaneous fat is removed by careful scraping and the skin is briefly rinsed in cold isotonic KCl. Skin disks are cut from the dorsal and lateral areas and are mounted on the skin penetration vessel by means of tightly fitting polyethylene rings. Any skin protruding beyond the edge of the rings after mounting on the penetration vessel is trimmed away, and a thin layer of paraffin is applied

around the edge. After the paraffin has hardened, it is coated with two applications of collodion or nail enamel to assure a water-tight seal.

The epidermal surface of the skin is immersed in either a water or buffer solution of the penetrating agent contained in the lower compartment of the skin penetration vessel. In experiments in which an enzyme is used to assess penetration, a suitable buffer at a pH for optimal enzyme activity is placed on both sides of the skin. The specific conditions (type of buffer used, pH, and molarity) are selected on the basis of the enzyme to be used, or the chemical penetrating the skin.

The penetration vessel, attached to the manometer, is placed in a constant temperature Warburg bath maintained at 37°C., and penetration rates are determined over a 2 to 4 hour period.

A list of some of the compounds studied and the reaction system used to determine their penetration is presented in Table I.

Table I

COMPOUNDS AND REACTION SYSTEMS USED TO DETERMINE SKIN PERMEABILITY

Compound	Reaction System	Manometric Measurement
Formic Acid	Potassium Bicarbonate	CO_2 Evolution
Lactic Acid	" "	" "
Glutamic Acid	Glutamic Acid Decarboxylase	" "
Histidine	Histidine "	" "
Urea	Urease	" "
Glucose	Glucose Oxidase	O_2 Uptake
n-Butylamine	Monoamine Oxidase	" "

Results and Discussion

A. Histology

The skin of rats on the EFA-deficient diet began to show alterations after 6 weeks, which became more marked after 16 and 20 weeks (Figures 1 & 2). The most obvious abnormality was the acanthosis or over-all general thickening of the epidermis with up to a 100% increase in thickness noted. Both the Malpighian and the granulosum layers showed 3-4 layers of cells instead of 1-2 layers, and the cells were packed with numerous irregular, basophilic-staining granules. The number of mitotic figures in the basal cells did not appear abnormal. The stratum corneum conjunctum was also thickened with parakeratosis evident. The acanthosis extended into the pilosebaceous duct and involved the hair follicles. The pilosebaceous duct openings were observed to be plugged with keratotic substance which extended down about one-third of the duct.

Another characteristic of EFA-deficient skin was the hyperplasia of the sebaceous glands. The acini of the glands were enlarged and were more numerous than in the control animals. In addition, the granular basophilic material appeared more prominent.

The prominence of the stratum granulosum in EFA-deficient skin was shown to good advantage under interference microscopy (Figure 3).

FIGURE 1. Skin Section of EFA-Deficient Rat - Giemsa Stain

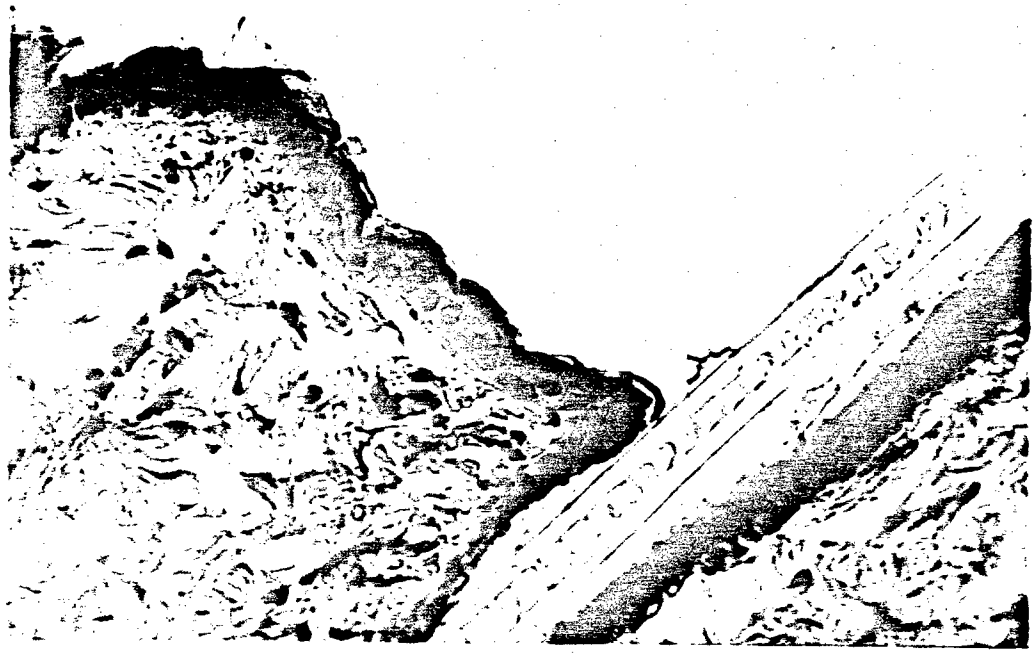


FIGURE 2. Skin Section of Control Rat - Giemsa Stain

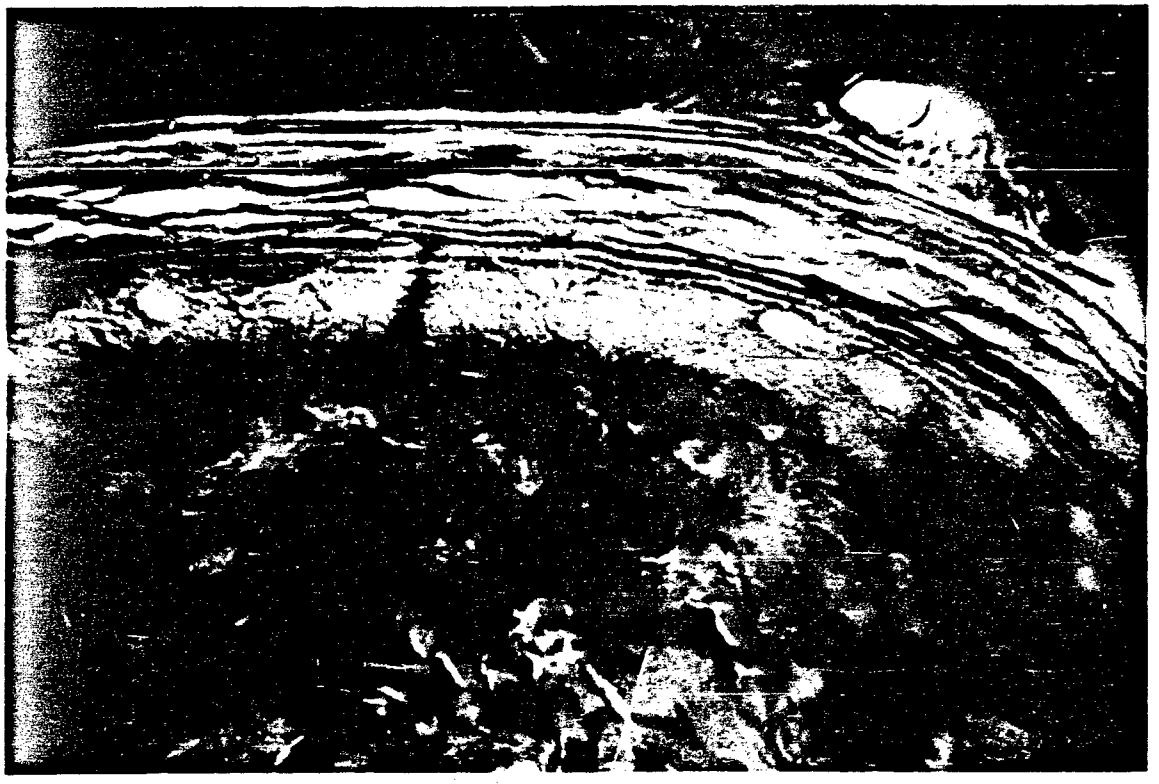


FIGURE 3. Skin Section of EFA-Deficient Rat by Interference Microscopy

The specimen is an unstained frozen section as seen with the interference microscope using monochromatic light, double focus 400X lens system, 238° analyzer setting and the one-quarter wave length plate.

Histological analyses of normal and deficient skins are given in Table II based on standard light and interference microscopy.

Table II

HISTOLOGICAL CHANGES IN SKIN OF EFA-DEFICIENT AND CONTROL RATS

Specimen Area	EFA-Deficient Rat	Control Rat
Stratum corneum	Compressed, closely packed; increased thickness of both conjunctum and disjunctum; basophile granules present	Relatively thin conjunctum and disjunctum layers
Stratum lucidum	Cannot be distinguished from stratum granulosum	One cell layer thick
Stratum granulosum	Thickened to 3-4 cell layers closely packed; numerous irregular granules	Normal thickness of approx. 1 cell layer; some irregular granules
Stratum germinativum	Thickened to 3-4 cell layers	Normal thickness of 1-2 cell layers
Pilosebaceous duct and follicle	Hyperplasia of duct lining	Normal epidermal thickness
Sebaceous gland	Hyperplasia with increased granulation	Normal
Dermis	Mononuclear infiltration proximal to epidermis	Normal
Subcutaneous tissue	No apparent change	Normal

B. Histochemistry and Chemistry

In view of the importance of protein sulfur in the keratinization process, and perhaps also in control of permeability, the sulfhydryl distribution of skin was visualized by a histochemical method, and the sulfhydryl and disulfide content of epidermis were determined by a chemical method.

Protein sulfhydryl groups have been classified (20) on the basis of whether they react with sulfhydryl reagents before or after denaturation of the protein, as "free" and "masked".

In the experiments discussed below, histochemical reaction with sulfhydryl was carried out after ethanol-trichloroacetic acid fixation, and probably only "free" sulfhydryl is demonstrated. In the chemical determinations of epidermal sulfhydryl, the tissue protein is first denatured with urea, in order to measure "total" sulfhydryl, i.e. both "free" and "masked".

Histochemistry

Of the various histochemical methods for demonstrating the presence of sulfhydryl groups in tissue, the method of Barrnett and Seligman using 2,2'-dihydroxy-6,6'-dinaphthyl-disulfide (DDD), is considered most specific (20). This method consists of two steps. DDD reacts by sulfide interchange with the free protein sulfhydryl, forming a colorless dithionaphthol derivative and liberating 6-thio-2-naphthol.

The colorless derivative is then converted to an intensely colored azo dye by coupling with tetrazotized diorthoanisidine (TDA). TDA is a bifunctional reagent, capable of reacting with 1 or 2 dithionaphthol residues. If the TDA couples with 1 residue, a red color is obtained; if it couples with 2 residues, a blue color is obtained. Thus both concentration and spatial arrangement of sulfhydryl groups with respect to each other are factors in color response. Absence of color indicates that sulfhydryl is not available.

Non-specific coloring of connective tissue (presumed to be sulfhydryl-free) may be the result of the reaction of TDA with histidine or tyrosine, or may possibly be due to incomplete extraction of the reagent.

The control animals exhibited low sulfhydryl concentration (light pink) in the corneum and dermal sheath of the hair. Somewhat higher sulfhydryl concentration occurred in the Malpighian layer, the external root sheath, and the papilla. The sebaceous glands also gave evidence of sulfhydryl groups. The dermal tissue and the hair shafts remained unstained, while the internal root sheath and the matrix of the bulb stained blue, indicating high sulfhydryl values (Figure 4).

The 16 week deficient animals showed higher levels of sulfhydryl groups in the epidermis, sebaceous glands, dermal sheath and external root sheath than were found in control skin.

The proximal area of the hair shaft developed a light blue stain, as did the papilla. The matrix and the external root sheath stained a dark blue and the corneum showed an even darker blue stain, indicative of heavy concentration of sulfhydryl groups.

In the 20 week deficient animals, the hair shaft was stained with shadings from red to blue. Sebaceous glands were intensely red; the dermal sheath, the external and internal root sheathes, the matrix, papilla and the epidermis all stained various intensities of blue. The corneum in these animals was stained so intensely as to appear almost blue-black in some sections (Figure 5).

Results on color reactions with the DDD reagent are given in Table III. Further histochemical studies of the early stages of the deficiency are in progress.

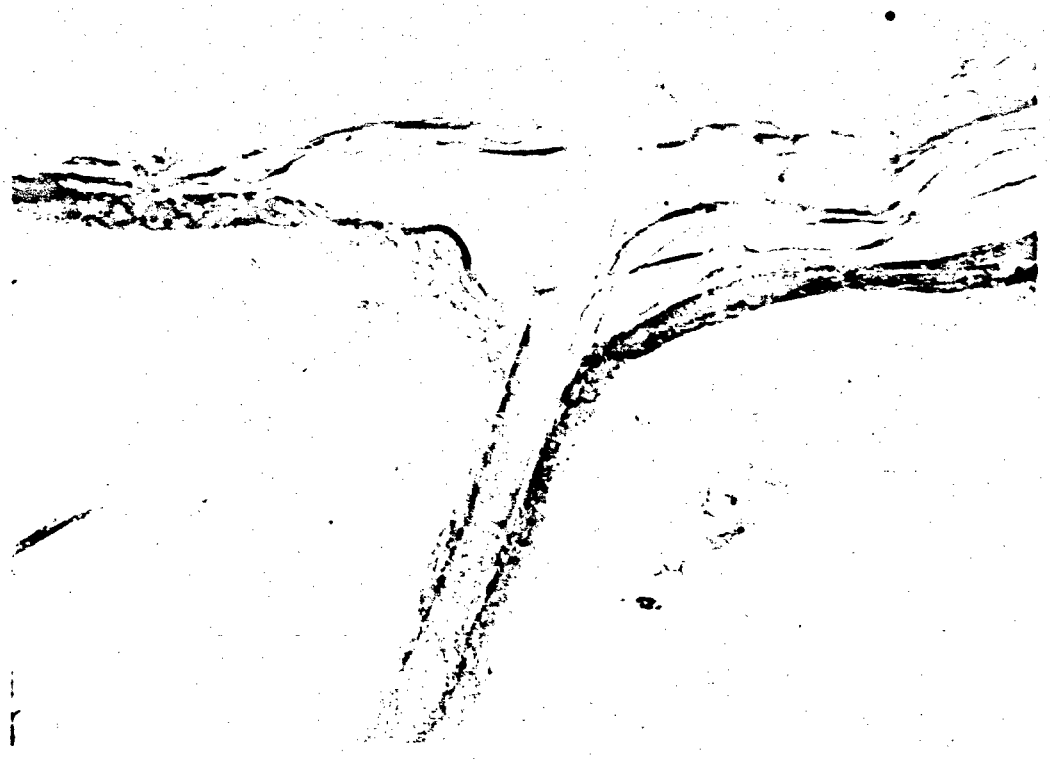


FIGURE 4. Skin Section of Control Rat - Barrnett and Seligman Reaction

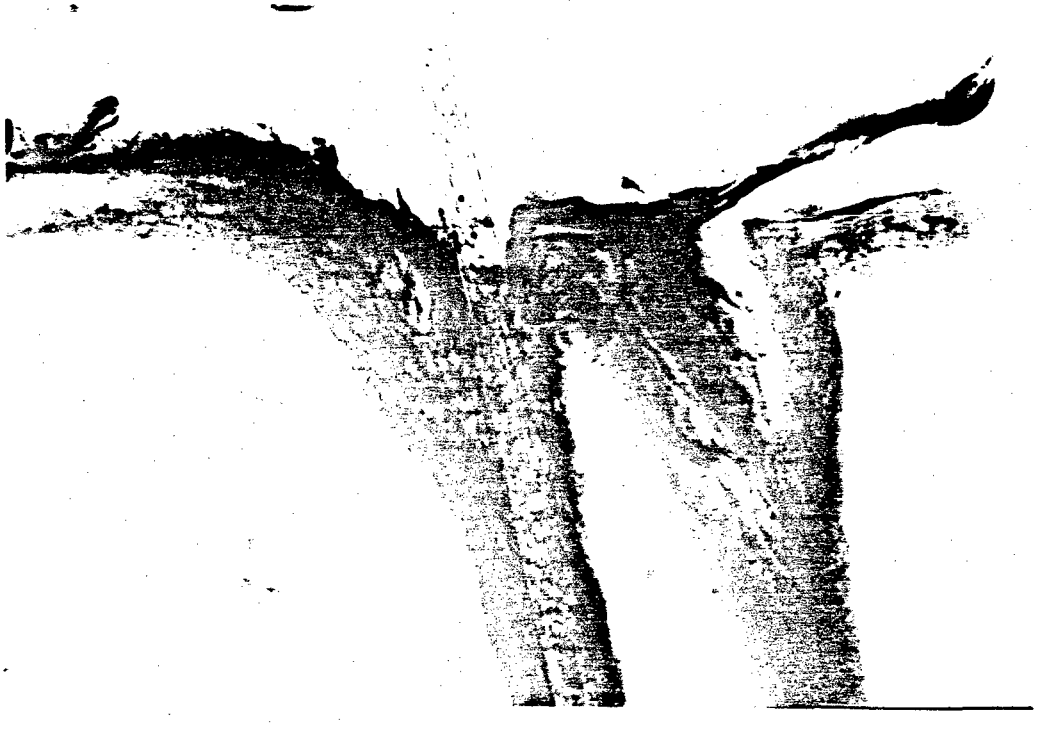


FIGURE 5. Skin Section of EFA-Deficient Skin - Barrnett and Seligman Reaction

Table III

RESULTS OF DDD STAINING OF SKIN OF CONTROL AND EFA-DEFICIENT RATS

Area of Skin	Control	16 Week EFA-Def.	20 Week EFA-Def.
Stratum Corneum	1	3	4
Malpighian Layer	1	2	3
Hair shaft	0	3 (proximal portion)	1-3 (entire shaft)
External root sheath	1	2	3
Dermal sheath	1	2	3
Matrix of bulb	3	3	3
Papilla	1	3	3
Sebaceous gland	1	2	2
Dermal connective tissue	0	0	0

0 = no reaction

1 = weak monocoupling: pink

2 = strong monocoupling: red

3 = weak dicoupling: light blue

4 = strong dicoupling: dark blue

Chemistry

In the chemical determination of sulfhydryl by the method of Flesch and Kun (17), 1-(4-chloromercuriphenylazo)-naphthol-2 (CMPAN) is employed as the sulfhydryl reagent. This reagent differs from the DDD-TDA system in that it combines with sulfhydryl groups only in a 1:1 (sulfhydryl:dye) ratio. In the use of this reagent, therefore, orientation of the sulfhydryl groups with respect to one another should play no part in the intensity or spectral properties of the color produced; availability of the groups for reaction is the only consideration. Availability for reaction of all or virtually all of the tissue sulfhydryl groups is believed to be effected by the incubation with 3M urea referred to above.

Total sulfur (cysteine plus cystine) determinations of epidermal proteins have been made after acid hydrolysis and evaporation under conditions that assure complete conversion of any cysteine present to cystine. Sodium naphthoquinone-4-sulfonate was reacted with the cysteine produced after reduction of cystine with cyanide, according to the method of Sullivan et al. (18). Disulfide sulfur was calculated by subtraction of sulfhydryl sulfur from total sulfur.

In Table IV are given the results of sulfhydryl and disulfide determinations carried out on epidermis of control and EFA-deficient rats.

Table IV

ANALYSIS OF EPIDERMIS OF CONTROL AND EFA-DEFICIENT RATS

Weeks on Diet	Sulfhydryl Sulfur mg./g. F.E.D.W.[a,b]	Sulfhydryl + Disulfide Sulfur[c] mg./g. F.E.D.W.	Disulfide Sulfur[d] mg./g. F.E.D.W.
Control	0.29 ± .08[e] (9)[f]	4.6 ± .8 (12)	4.3
16	0.34 ± .06 (5)	4.8 ± 1.5 (8)	4.4
20	0.53 ± .22 (5)	5.8 ± 1.8 (4)	5.3

[a] F.E.D.W. = Fat-extracted dry weight

[b] SH values corrected to F.E.D.W. on basis of loss in dry weight following TCA-precipitation and ethanol-ether extraction of fat. Recovery factors employed: control, 74.9 ± 8.3%; experimental, 16 wk., 85.8 ± 4.9%, 20 wk., 88.6 ± 7.9%.

[c] Determined as cystine

[d] Calculated by difference

[e] Means and standard deviations

[f] No. of rats

The data in Table IV show that no statistically significant change occurred in total protein sulfur (cysteineplus cystine) content during the course of the experiment, and that a significant increase in the total sulfhydryl content of deficient epidermis became evident only after 20 weeks. Histochemically, an increase in sulfhydryl content of deficient epidermis was seen

at an earlier period (16 weeks). In the light of the different specificity (discussed above) of the chemical and histochemical methods, the results are not incompatible.

Further chemical analyses of EFA-deficient skin are in progress.

C. Penetration and Permeability Studies

Water Diffusion and Electrical Conductivity

The high water loss through the skin of EFA-deficient animals was further investigated to determine whether it could be correlated with changes in electrical conductivity of the skin. The latter measurement is considered an index of the ability of ions to pass through the barrier region of the epidermis.

Results are given in Table V.

The electrical conductivity of the skin of the EFA-deficient rats was somewhat higher than that of the controls after 3 weeks on the deficiency diet. A sharp increase in conductivity occurred between the 6th and 10th weeks of the experiment. Similarly, the water diffusion rate increased sharply during this period. The constancy in the ratio of diffusion rate to conductivity, particularly in the experimental groups, would indicate that the same barrier operates for controlling water diffusion and passage of ions through skin.

Table V

ELECTRICAL CONDUCTIVITY (I) AND WATER DIFFUSION RATE (DR)

OF SKIN OF CONTROL AND EFA-DEFICIENT RATS

Weeks on Diet	No. of Rats	I μamp/volt		DR (24 Hour Period) mg./cm.^{2}/hr.		Ratio DR/I	
		Control	Exptl.	Control	Exptl.	Control	Exptl.
3	10 C, 9 E**	1.8 ± 0.7*	2.8 ± 1.2	-	-	-	-
5	10 C, 10 E	1.3 ± 0.6	2.6 ± 1.3	-	-	-	-
6	4 C, 5 E	1.7 ± 0.5	3.4 ± 1.2	0.24 ± 0.05	0.28 ± 0.17	0.14 ± .03	0.08 ± .03
10	4 C, 7 E	0.8 ± 0.3	17.0 ± 4.3	0.14 ± 0.02	1.16 ± 0.24	0.18 ± .07	0.07 ± .02
12	6 C, 7 E	0.7 ± 0.3	14.9 ± 2.8	0.17 ± 0.07	1.12 ± 0.14	0.29 ± .14	0.08 ± .02
15	6 C, 7 E	0.7 ± 0.1	15.2 ± 1.3	0.27 ± 0.12	1.34 ± 0.29	0.27 ± .14	0.09 ± .01

* Means and Standard deviations

** C = control, E = experimental

The sharp increase in permeability of deficient skin to water and ions which occurs between the 6th and 10th weeks of the deficiency state is being further investigated.

The Penetration of Organic Compounds

In the last semi-annual report (7) some preliminary studies on the penetration of several chemicals through normal and EFA-deficient rat skin were presented. The results indicated that normal, intact rat skin was not permeable to sulfuric, lactic and glutamic acid, whereas formic acid was able to penetrate quite readily. EFA-deficient rat skins were permeable to all four substances. These studies have been extended and the penetration of a number of additional compounds through normal and EFA-deficient rat skin has been investigated.

Organic Acids

Subsequent studies with normal and EFA-deficient rat skins have confirmed the initial observations that formic acid will penetrate both types of skins readily. However, the rates of penetration of formic acid showed considerable variability, with values ranging from 0.37 to 1.3 micromoles/ $cm.^2/hr.$ for normal rat skin, and from 4.4 to 13.2 micromoles/$cm.^2/hr.$ for EFA-deficient skin. In all experiments the penetration rates were from 5 to 10 times greater through EFA-deficient skin than through normal.

The other organic acids studied in this series of experiments were acetic, propionic and n-butyric. The rates of penetration of these compounds through normal and EFA-deficient skin were of the same magnitude and as variable as the rates observed with formic acid.

The results are presented in Table VI.

Table VI

PENETRATION OF ACETIC, PROPIONIC AND BUTYRIC ACIDS THROUGH NORMAL AND EFA-DEFICIENT RAT SKIN

	Penetration Rate (Micromoles/cm.^{2}/hr.)			
	Normal Skin	(Range)	EFA-Def. Skin	(Range)
Acetic Acid (.5N)	0.53	(.08 - 1.3)	4.27	(3.6 - 8.5)
Propionic (.5N)	0.81	(.70 - .96)	6.07	(2.7 - 8.2)
Butyric Acid (.5N)	1.05	(.88 - 1.21)	-	-

One of the objectives of these experiments was to determine possible differences in penetration rates among these compounds, but because of the variability in rate values, no significant differences could be distinguished. Possible rate differences cannot be ruled out, however, since the variable results may have been due to certain technical difficulties encountered at the time these studies were performed. Since then, the experimental procedure has been modified, and the penetration of these compounds is being reinvestigated under the improved experimental conditions.

Previously reported studies did not indicate any significant penetration of lactic acid through normal rat skin during a one hour exposure. In subsequent studies where the experimental period was extended to three hours, a significant penetration rate of approximately 0.30 micromoles/cm.^{2}/hr. could be detected. Similar to the results obtained with the other organic acids studied, the penetration of lactic acid through EFA-deficient rat skin was about 10 times greater than through normal skin.

Amino Acids

The results previously reported, that no significant penetration of glutamic acid could be detected through normal rat skin, have been confirmed in several additional experiments. With respect to EFA-deficient skin some penetration could be detected, but the rate was low compared to the other compounds

studied. The penetration of one other amino acid, histidine, was evaluated. As with glutamic acid, no significant penetration occurred through normal rat skin. The permeability of EFA-deficient skin to histidine has not been determined.

Glucose

Penetration of glucose could be detected through neither normal nor EFA-deficient skin. Because of the low activity of glucose in contrast to other compounds, it was of interest to determine how readily glucose would penetrate skin from which the epidermis had been removed. The results presented in Table VII show that ammonia-separated dermis is highly permeable to both formic acid and urea, but that even under these conditions, glucose penetration is considerably slower.

Table VII

PENETRATION RATES OF GLUCOSE, FORMIC ACID AND UREA THROUGH AMMONIA-SEPARATED DERMIS FROM NORMAL RATS

	Concentration	Penetration (μmoles/cm.^{2}/hr.)
Glucose	0.5M	1.69
		1.82
Formic Acid	0.33M	18.4
		16.7
Urea	0.5M	22.7

Urea

The penetration of urea was determined through both normal and EFA-deficient rat skin. In the presence of urease in the dermal chamber of the penetration vessel and no urea on the other side of the skin, an average of 166 microliters of CO_2 were evolved over a two hour period. When urea was added to the epidermal side of the skin, CO_2 evolution over a two hour period averaged 200 microliters. The net CO_2 evolution of 34 microliters corresponds to a urea penetration rate through normal skin of approximately 0.02 micromoles/cm.^{2}/hr., indicating very little urea penetration through normal rat skin. Urea penetration determined on skins of two different groups of EFA-deficient rats gave average rates of 2.16 and 3.62 micromoles/cm.^{2}/hr. These results are presented in Figure 6.

Urea has a unique penetration pattern in the sense that it does not pass through normal skin but readily penetrates EFA-deficient skin. Organic acids penetrate readily EFA-deficient skin, but also penetrate normal skin. Glucose and glutamic acid, like urea, do not penetrate normal skin but, unlike urea, penetrate EFA-deficient skin at a low rate.

The rates for these different compounds through normal and EFA-deficient skin are summarized in Table VIII. The rates were calculated from the linear portion of the slopes.

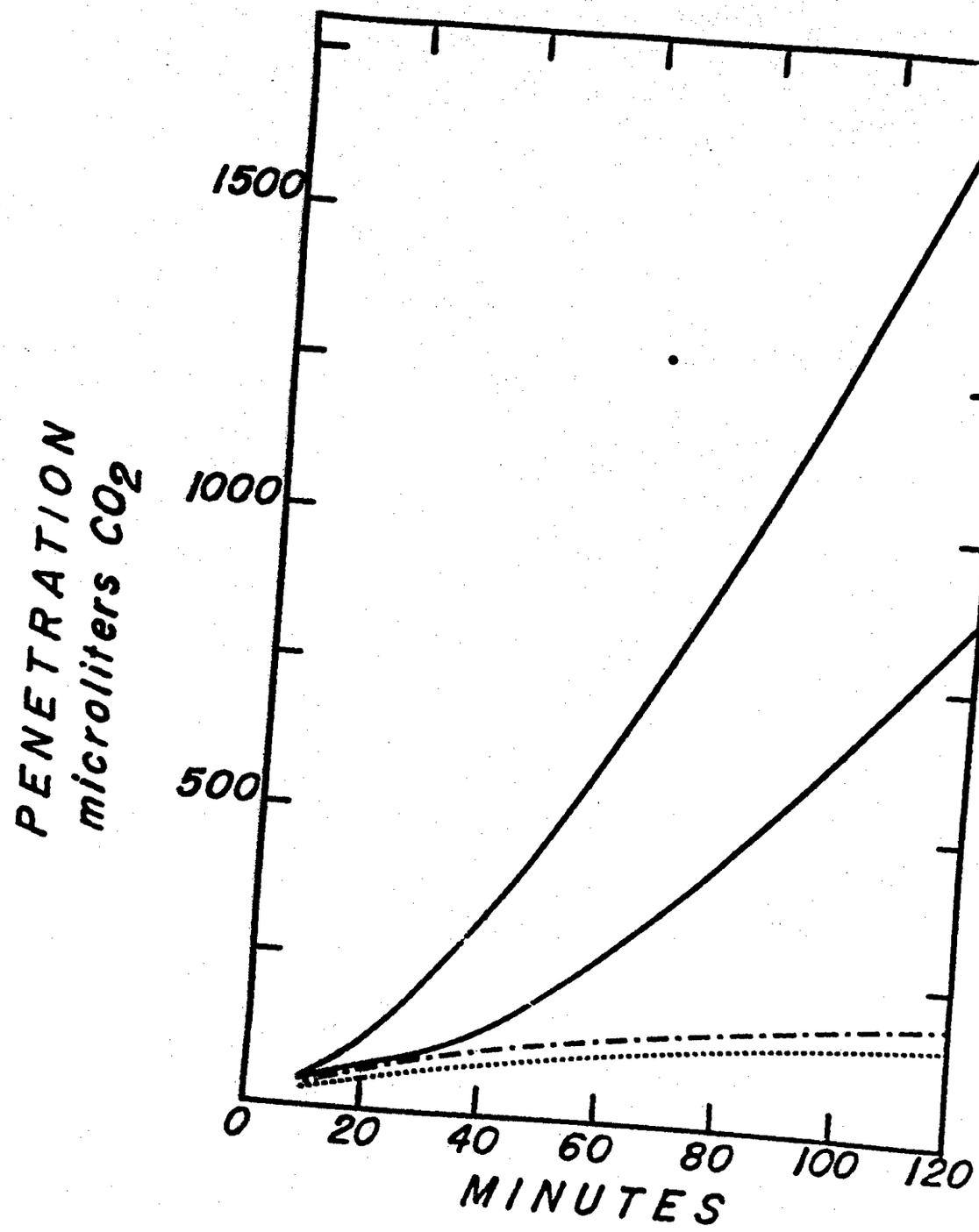


FIGURE 6. Urea Penetration through Skins from Normal and EFA-Deficient Rats.

EFA-Deficient: ——;
Normal Controls: —·—;
Enzyme Blank: ·····.

Table VIII

PENETRATION RATES OF VARIOUS CHEMICALS THROUGH NORMAL AND EFA-DEFICIENT RAT SKIN

Substance	Penetration Rate (μmoles/cm.^{2}/hr.)	
	Normal Skin	EFA-Deficient Skin
Formic Acid (.33N)	0.94	9.35
Lactic Acid (.2N)	0.30	3.67
Glutamic Acid (.2N)	0.04	0.25
Glucose (.25M)	$<$ 0.02	$<$ 0.05
Urea (.5M)	0.02	2.16, 3.62

Because of its low penetration through normal skin and the high penetration observed through EFA-deficient skin, urea is being employed as a standard agent for investigating possible alterations in skin permeability as a result of experimentally induced stresses applied to skin as described in other sections of this report.

Nickel Penetration

The penetrability of nickel chloride, alone and in the presence of sodium lauryl sulfate (NaLS) was investigated in the skin of two EFA-deficient and two control animals. The presence of nickel in the skin was demonstrated by both histochemical and autoradiographic methods. The results are given in Table IX.

Nickel chloride alone did not appear to penetrate control skin beyond the stratum corneum, whereas it was found to greater depth in the epidermis of the deficient skin. Histochemical and autoradiographic observations indicated that in the presence of sodium lauryl sulfate, nickel penetrated deficient skin and control skin equally well. In the presence of the surfactant, nickel was observed to be present in the Malpighian layer. Whether it gained entry through the barrier or entered the basal layer from the dermis is not known. The presence of high concentrations of nickel in the pilosebaceous duct would indicate this route as a principal pathway for entry of nickel into the dermis.

Table IX

NICKEL PENETRATION OF NORMAL AND EFA-DEFICIENT SKIN

Skin	Treatment	Nickel Penetration of Skin
Control	$Ni^{63}Cl_2$, 0.5%	Ni present in stratum corneum. Some in the pilosebaceous orifice.
EFA-Deficient	$Ni^{63}Cl_2$, 0.5%	Ni present throughout the epidermis. Some in the orifice of the pilosebaceous duct.
Control	$Ni^{63}Cl_2$, 0.5% and NaLS, 0.5%	Ni present throughout the epidermis, upper dermis and pilosebaceous duct.
EFA-Deficient	$Ni^{63}Cl_2$, 0.5% and NaLS, 0.5%	Ni present throughout the epidermis, upper dermis and pilosebaceous duct.

III. SKIN LIPID ANALYSES

The essential fatty acid (EFA)-deficiency syndrome of rats has been the object of intensive study by numerous investigators since it was first recognized by Burr and Burr in 1929 (1); yet little has been learned of the molecular functions or the sites of action of the essential fatty acids. The literature on the subject has recently been reviewed by Aaes-Jorgensen (5), who suggested that EFA may be of importance for the structure of cell membranes and, therefore, may be related to membrane permeability. He points out, however, that the reasons why a few of the polyenoic acids are essential are not well understood at present.

We have begun a comparative analysis of the lipid profiles of the skins of normal rats and the skins of EFA-deficient rats using thin layer and gas-liquid chromatography. Preliminary results obtained during the course of setting up the analytical procedures are shown in Figures 7 and 8.

Figure 7 is a chromatogram of two lipid mixtures extracted from rat skin and of certain known lipids (glycerol, cholesterol, stearic acid, triolein, a mixture consisting essentially of saturated mono- and diglycerides, and

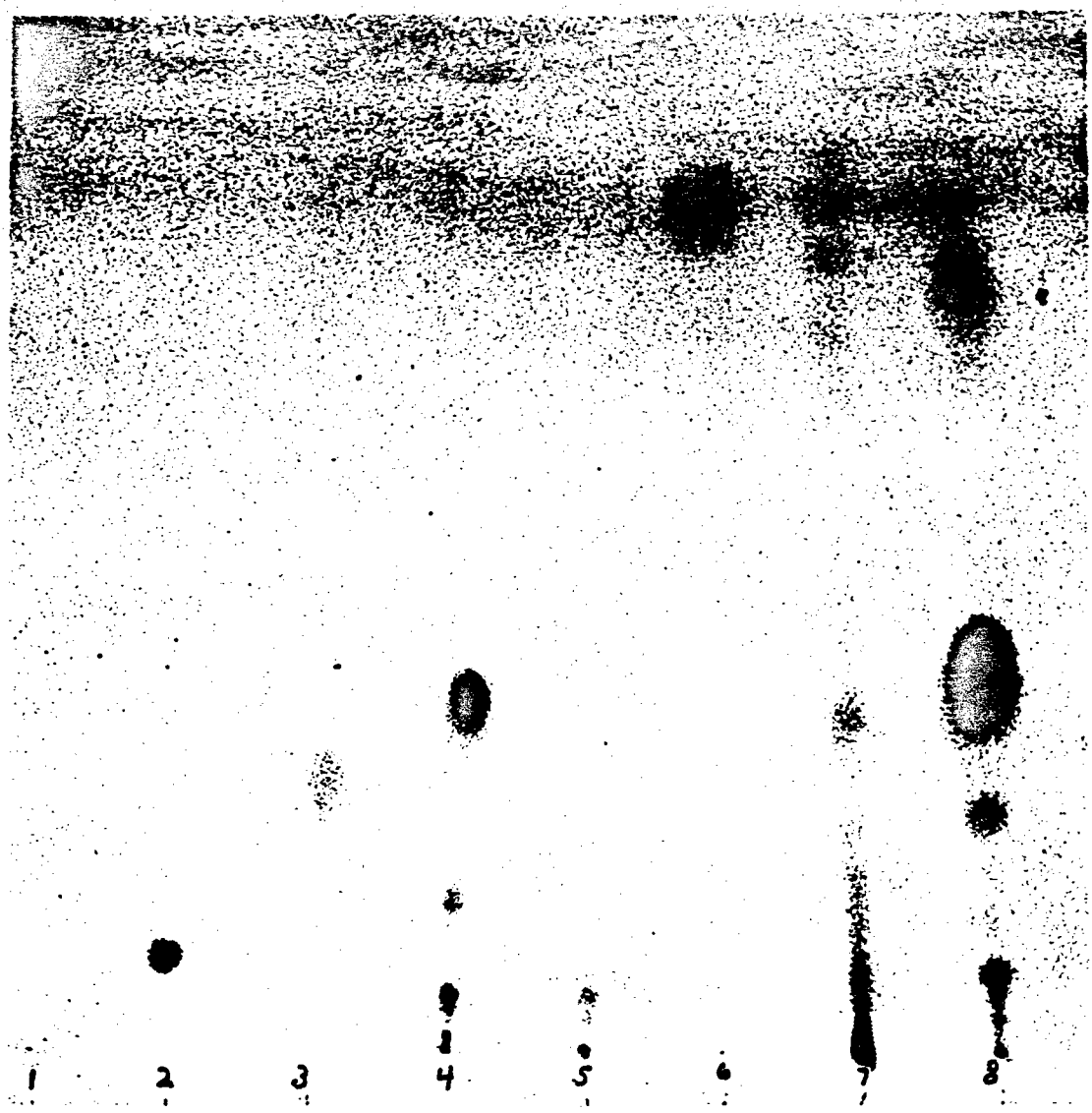


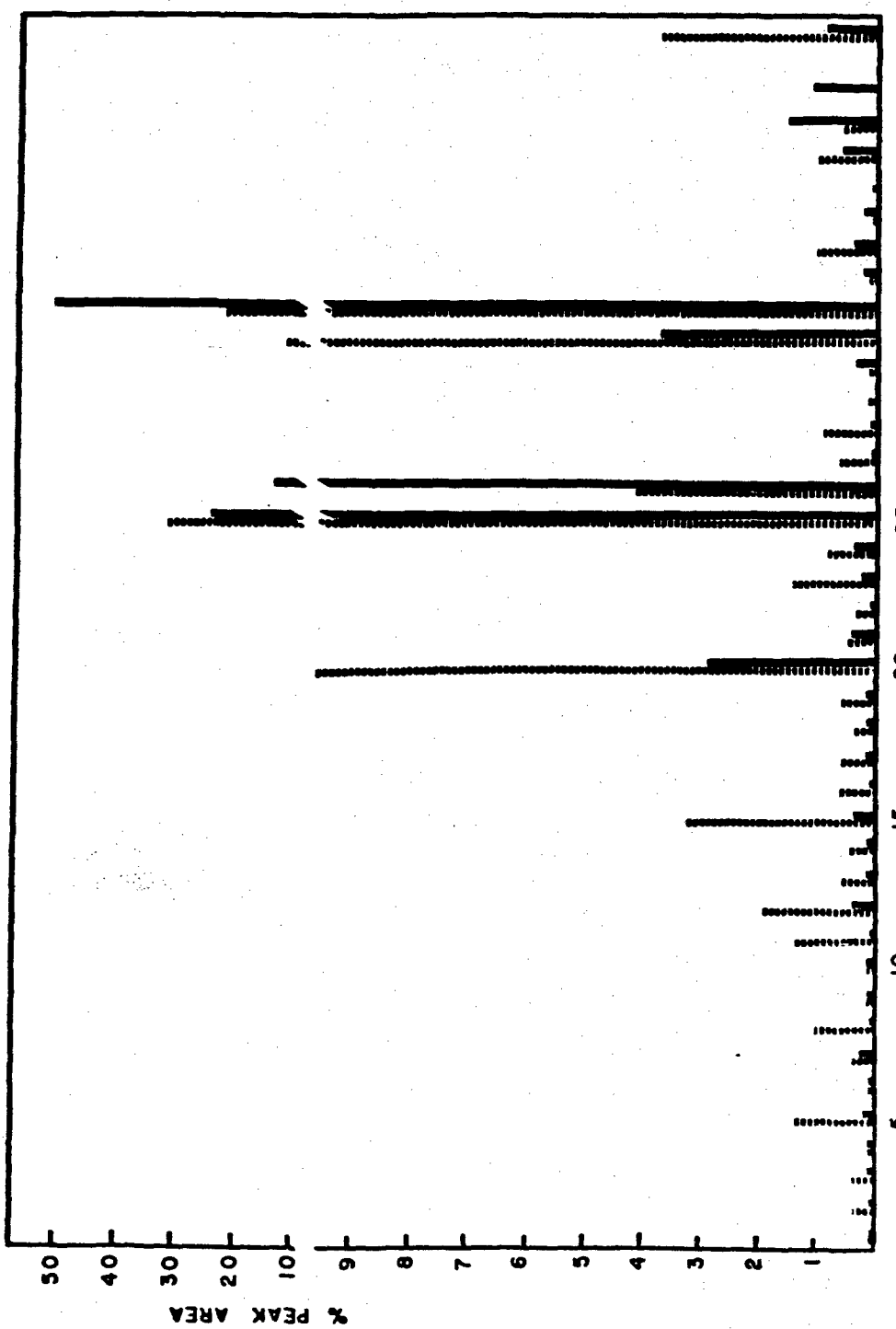
FIGURE 7

8142-60 Thin Layer Chromatogram of Lipid Substances
Adsorbent: Silica Gel G, 250 μ
Eluant: Pet. ether/90: ethyl ether/10: glac. HOAc/1
Developer: Phosphomolybdic acid

1. Glycerol, 20γ
2. Cholesterol, 20γ
3. Stearic acid, 20γ
4. Triolein, 20γ
5. Mixed glycerides E-16, 20γ
6. Squalane, 20γ
7. Normal rat whole skin lipids, approx. 150γ
8. EFAD rat skin lipids, approx. 150γ

squalane) chromatographed on a thin layer of Silica Gel G according to the method of Stahl (21). The eluant was petroleum ether:ethyl ether:acetic acid (90:10:1) (22). The lipid spots were developed with phosphomolybdic acid (23). The intensity of staining is variable because conditions for optimal color development depend on the material involved. Under the conditions used here (heating 30 minutes at 105°) glycerol (which does not move in this system) and the saturated glycerides are only weakly stained. The triolein used in this run was found to have at least two slower moving components, possibly mono- and diolein. The last two samples are mixtures of skin lipids obtained from one control and one EFA-deficient rat. The skin, from which hair, fascia, and subcutaneous fat had been removed, was homogenized, lyophilized and extracted for two days with petroleum ether. The patterns observed have been reproduced on other runs using the same samples. There are obvious differences between the lipid pattern of the control rat and of the EFA-deficient rat but interpretations must be deferred until additional animals are studied.

Figure 8 is a gas chromatographic analysis of the total fatty acids obtained from the skin of one EFA-deficient rat and one control rat. These two samples are the same ones analyzed by thin layer chromatography (Figure 7).



GAS CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS DERIVED FROM WHOLE RAT SKIN

——— EFA-DEFICIENT ..... CONTROL

FIGURE 8

The analyses were performed on a Barber-Colman Model 10 fitted with an 8 ft. column and a flame ionization detector. The stationary phase was 20 per cent ethylene glycol - glutaric acid resin on 80-100 mesh Chromosorb W. Column temperature was maintained at 170°C. The analysis was carried out on the methyl esters of the fatty acids. The lipid was saponified by refluxing the sample in 15 ml. of 0.5 N KOH in methanol for one hour. The mixture was diluted with water, extracted five times with petroleum ether to remove the unsaponified fraction, acidified with hydrochloric acid and again extracted with petroleum ether to isolate the fatty acids. Refluxing in methanol, containing a catalytic amount of sulfuric acid, converted the acids to the methyl esters which were isolated by dilution with water and extraction with petroleum ether. The peaks in Figure 8 are numbered in order of elution and the major components have been tentatively identified by the usual convention where C18:1, for example, represents the straight chain 18-carbon acids containing one double bond - in this case largely oleic acid.

While it must be emphasized again that these are preliminary results on only two animals we wish to point out certain differences between the two samples. The lipids isolated from the skin of the EFA-deficient animal are relatively much richer in the monoenoic acids of 16-, 17-,

18-, 19-, and 20-carbon chain length (peaks 26, 30, 32, 35, and 38) than those isolated from the control. In fact the C18:1 fraction accounts for 49 per cent of the fatty acids of the EFA-deficient rat and only 21 per cent of the fatty acids of the control. The control lipid is correspondingly richer in the other major fractions including the C18:2 and C20:3 (tentative identification) fractions. Of particular interest is peak 39 which has tentatively been identified as the C20:2 fraction. This component represents about one per cent of the fatty acids of the EFA-deficient sample and was not detected in the control sample.

These two methods will be combined to obtain a more detailed analysis of the skin lipids in the epidermis and the dermis as well as in the whole skin. Thin layer chromatography will separate the lipids into fractions such as hydrocarbon, sterol, mono-, di- and triglycerides, and unesterified fatty acid. The fatty acid composition of any fraction can then be analyzed by gas chromatographic methods.

IV. PENETRATION STUDIES ON SKIN OF ANIMALS MADE NUTRITIONALLY DEFICIENT IN FACTORS OTHER THAN EFA

Studies have been carried out to ascertain the effects on skin permeability of dietary deficiencies known to cause morphologic alterations of the skin. The conditions chosen were deficiencies in vitamins A, B_6, and C, and in magnesium. The essential nutrients studied have certain distinctive effects on skin: vitamin A is involved in normal germinative layer differentiation (24); vitamin B_6 is necessary for normal fat metabolism (4); vitamin C is essential for maintaining the integrity of connective tissue (25); magnesium deficiency results in epidermal lesions (26). In addition, protein deprivation was investigated as to its effect on epidermal integrity.

Effects of the deficiency conditions on skin permeability were assessed by determinations of water diffusion rate, electrical conductivity (as a measure of ion migration), and urea penetration rate.

Data on the animals and experimental conditions are given in Table X.

In Table XI are given the results of electrical conductivity measurements and water diffusion rate determinations on the skin of animals subjected to the experimental conditions described above.

Table X

EXPERIMENTAL CONDITIONS AND GROSS OBSERVATIONS

Dietary Regimen	Strain	Sex	Average Initial Body Wt. g.	Time on Diet weeks	Deficiency Symptoms
Vitamin A deficiency[1]	Sprague-Dawley Rats	M,F	50	9	Retarded growth, high spontaneous mortality.
Vitamin B_6 deficiency[2]	Sprague-Dawley Rats	M,F	53	8	Retarded growth, scaling of skin of ears,paws,nose,jaw.
Vitamin C deficiency[3]	Lever Colony Guinea Pigs	M	365	4	Weight loss, skin lesions
Magnesium deficiency[4]	Hemlock Hollow Farms Rats	M	54	8	Weight loss, sores over entire body surface.
Protein deficiency and excess[5]	Hemlock Hollow Farms Rats	M,F	47	13	Retarded growth, scaliness of skin over entire body.

[1] "Vitamin A and Ascorbic Acid Deficient Test Diet" (Nutritional Biochemicals Corp.). Controls fed same diet supplemented with 20,000 units of vitamin A per kg. of diet.

[2] B_6-deficient diet of Cerecedo *et al*. (27). Controls fed same diet supplemented with 4 mg. pyridoxine per kg. diet.

[3] "Ascorbic Acid Deficient Diet (Guinea Pig)" (Nutritional Biochemicals Corp.). Controls fed same diet supplemented with 1 g. ascorbic acid per kg. of diet.

[4] "Low Magnesium Test Diet" (Nutritional Biochemicals Corp.). Normal animals maintained on Rockland rat pellets were employed as controls.

[5] a) "Low 8% Protein Diet"; diet contains 8% casein; b) "High Protein Diet"; diet contains 64% casein; c) "Normal Protein Test Diet"; diet contains 27% casein (Nutritional Biochemicals Corp.).

Table XI

ELECTRICAL CONDUCTIVITY (I) AND WATER DIFFUSION RATE (DR) MEASUREMENTS OF SKIN OF ANIMALS ON VARIOUS DIETARY REGIMENS

Diet Group	Test Animal	No. of Animals	I $\mu amp/volt$	DR $mg./cm.^2/hr.$
A-Deficient	Rat	10	0.5 ± 0.2*	0.25 ± 0.05
A-Control	Rat	10	0.6 ± 0.2	0.26 ± 0.10
B_6-Deficient	Rat	10	0.6 ± 0.3	0.14 ± 0.03
B_6-Control	Rat	9	1.3 ± 0.6	0.20 ± 0.06
C-Deficient	Guinea pig	5	0.7 ± 0.2	0.20 ± 0.03
C-Control	Guinea pig	4	1.0 ± 0.3	0.32 ± 0.06
Mg-Deficient	Rat	7	1.6 ± 1.0	0.20 ± 0.12
Mg-Normal	Rat	5	1.3 ± 0.6	0.32 ± 0.06
Low Protein, 8%	Rat	6	2.3 ± 1.0	0.35 ± 0.18
Normal Protein, 27%	Rat	6	2.5 ± 1.2	0.19 ± 0.04
High Protein, 64%	Rat	6	2.0 ± 0.7	0.35 ± 0.16

*Means and standard deviations

The data in Table XI show that in vitamin B_6 deficiency, both electrical conductivity and water diffusion rate are slightly, but significantly decreased, as compared to values for supplemented controls. At low and very high dietary protein levels, water diffusion rates are increased as compared to values obtained with a normal protein level. However, because the magnitude of these changes is small, little importance is attached to the observations.

The results of urea penetration studies on skin from rats made deficient in vitamin B_6, vitamin A, or protein, are shown in Figure 9. There are no significant differences between the rates observed for deficient and normal skin.

Although the results presented above are largely negative, it is of interest that, even in the presence of macroscopic lesions of the skin, permeability remains essentially unchanged.

It is particularly surprising that no permeability increase is seen in vitamin B_6 deficiency, in view of the profound effect of this deficiency state on fat metabolism (4). There can be no doubt about this conclusion, however, since three different methods were employed for assessment of permeability.

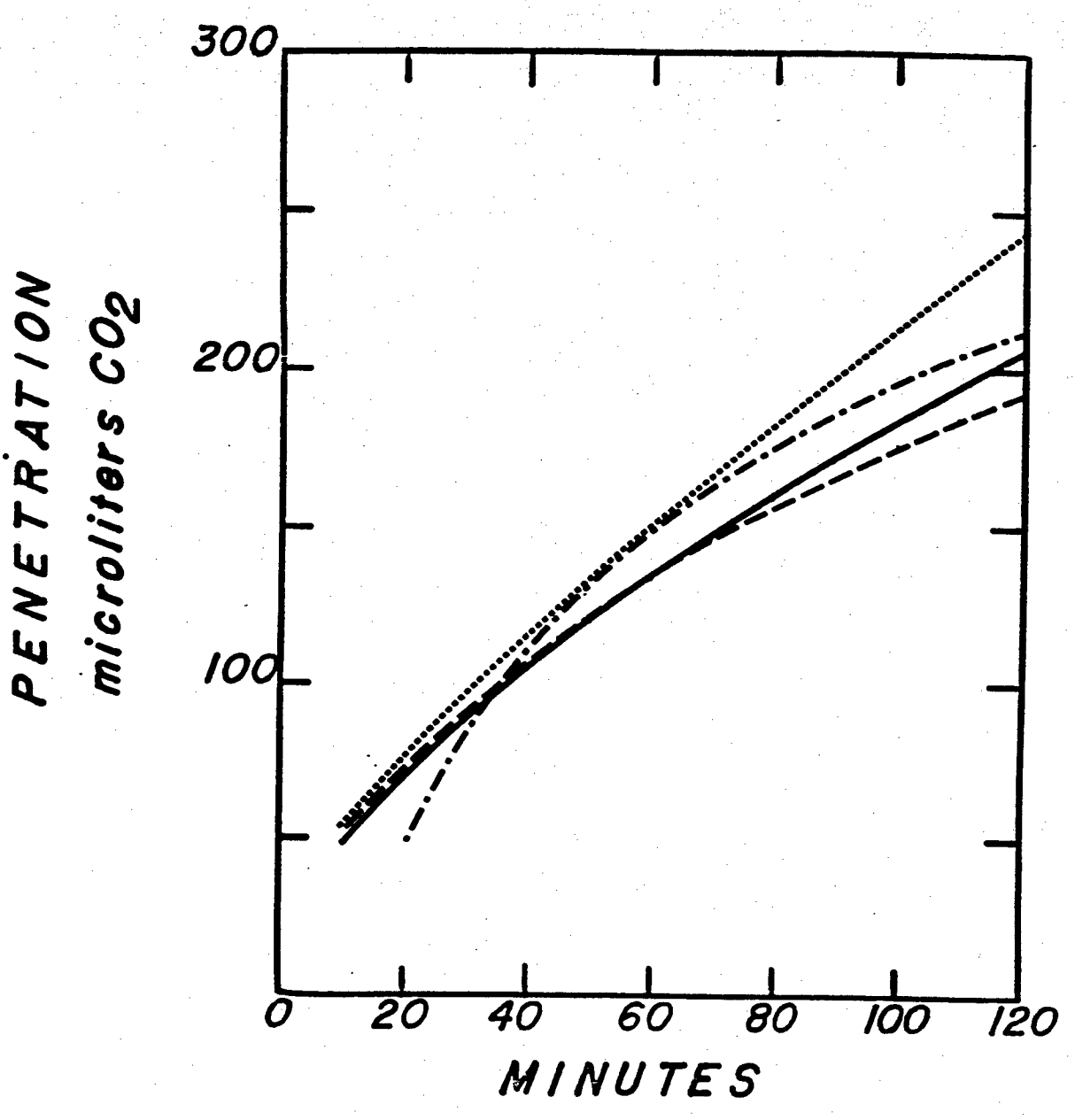


FIGURE 9. Penetration of Urea through Skins from Rats with Various Nutritional Deficiencies.

Low Protein Diet: .....;
Vitamin B_6-Deficient Diet: —·—·;
Vitamin A Deficient: — — —;
Normal Control: ———.

V. EFFECT OF SOLVENTS ON SKIN PERMEABILITY

The effect of organic solvents on the permeability of skin was studied employing the standard criteria of electrical conductivity, water diffusion rate, and urea penetration rate.

A. Electrical Conductivity and Water Diffusion Rate Measurements

Male rats, about 210-260 g. (Hemlock Hollow Farms) were used. They were maintained on a stock diet of Rockland rat pellets and water, available ad libitum.

The various solvents, viz. 95% ethanol, olive oil, petroleum ether, acetone, and a water control, were applied by rubbing the clipped flanks 10 times with a piece of cotton saturated with the solvent. This operation was repeated for a total of 5 times.

The apparatus for and method of determining electrical conductivity and water diffusion rate are described in Section II. Electrical measurements, with both electrodes placed on the solvent treated area, were taken immediately before and one-half hour after treatment. Each animal was sacrificed by cervical fracture one

hour after rubbing with solvent, and the treated skin excised and mounted on the water diffusion chamber. The data obtained are given in Table XII.

Table XII

ELECTRICAL CONDUCTIVITY AND WATER DIFFUSION RATE MEASUREMENTS OF SOLVENT TREATED* RAT SKIN

Solvent	No. of Animals	Electrical Conductivity (I) μamp/volt	Water Diffusion Rate (DR) mg./cm.^{2}/hr.
Water	8	1.8 ± 0.5**	0.30 ± 0.06
Ethanol, 95%	8	2.0 ± 0.7	0.38 ± 0.13
Olive oil	8	3.4 ± 1.7	0.55 ± 0.14
Petroleum ether, B.P. 30-60°C.	8	4.8 ± 2.4	0.54 ± 0.18
Acetone	8	20.6 ± 7.5	1.80 ± 0.48

* Before treatment the electrical conductivity value was 1.4 ± 0.6 μamp/volt

** Means and standard deviations

Under the experimental conditions employed, there is no significant difference between treatments with water and with 95% ethanol. Olive oil and petroleum ether treatments result in slight but significant increases in the conductivity and water permeability of skin. A pronounced change is observed only after exposure to acetone.

B. Urea Penetration Through Acetone Treated Rat Skin

The effect of acetone treatment of normal rat skin on urea penetration has been investigated in two experiments. In the first experiment, the dorsal area of a rat clipped free of fur was patched for 30 minutes with gauze which was kept saturated with acetone. The animal was sacrificed and skin disks were mounted on the penetration vessel. In the second experiment, skin from the trunk of a normal rat, clipped free of fur, was freed of subcutaneous fat and immersed in 200 ml. of acetone for 10 minutes with intermittent agitation. At the end of this period, the skin was removed and the excess acetone was evaporated from the skin, and skin disks were mounted on the vessels.

Urea penetration through the acetone-treated skins was determined by means of the urease reaction. The total CO_2 evolution over a two-hour period was 354 microliters for the 30 minute acetone patched skin and 404 microliters for the 10 minute acetone treated skin (Figure 10). Taking into account the 166 microliters of CO_2 produced by the action of urease on untreated rat skin in the absence of urea, the calculated urea penetration rates through these two skin samples are 0.08 micromoles/cm^2/hr. for the acetone patched skin, and 0.10 micromoles/cm^2/hr.

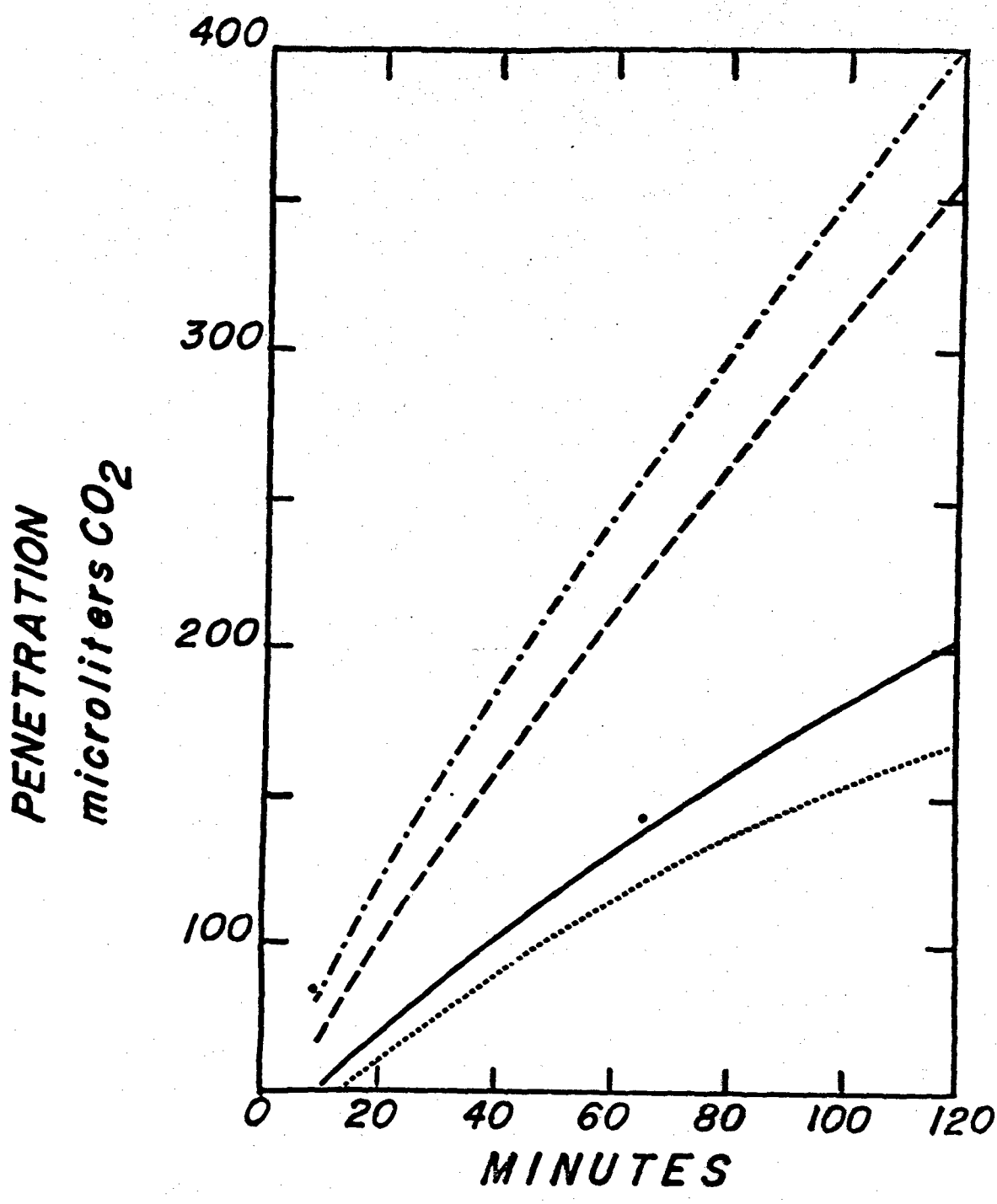


FIGURE 10. Urea Penetration through Acetone-Treated Skin.

The Rate of CO_2 Evolution is Plotted for:

30 Minute Acetone Patched Skin: — — —;
10 Minute Acetone Extracted Skin: — · — · —;
Untreated Normal Rat Skin: ———;
Enzyme Blank: ····· .

for the acetone extracted skin. Comparing these values with the average urea penetration rate of 0.02 micromoles/cm^2/hr. through normal, untreated skin would indicate that the acetone treatments resulted in a definite increase in skin permeability to urea.

C. Histology

Sections of skins from rats sacrificed one hour after the solvent treatments were processed for histological analysis. The following observations were made:

a) Water, olive oil and alcohol had no adverse effect on the skin.

b) Sections of skin treated with petroleum ether showed a well defined granulosum layer, 2-3 cells thick.

c) Acetone-treated skin gave evidence of deep seated penetration by the solvent. Alteration of the stratum granulosum was marked, showing strongly basophilic staining granules 2-3 layers deep. In the Malpighian layer, the basal columnar cells were in many instances flattened.

VI. THE PENETRATION OF AMINES THROUGH NORMAL SKIN

One of the objectives of the skin permeability studies is to investigate the effect of chemical structure on penetration rates through skin. By determining the penetration rates through skin of a homologous series of amines the effect of such variables as carbon chain length, molecular weight, and substituent groups on skin permeability could be evaluated. By using an enzyme, monoamine oxidase, the penetration of amines through skin can be readily determined manometrically by measuring oxygen consumption resulting from the overall reaction:

$$RCH_2\text{-}NH_2 + 1/2\ O_2 \xrightarrow{MO} RCHO + NH_3$$

It is planned in the course of these studies to investigate the penetration rates of a series of aliphatic amines from methylamine to octylamine, and also a number of aromatic amines such as phenylethylamine and various hydroxy-substituted derivatives (adrenaline and tyramine).

While monoamine oxidase is found in most organ tissue, a wide variability in substrate activity exists depending on the organ and animal from which the enzyme is extracted. The greatest range in activity with respect to the number

of amines oxidized is exhibited by the enzyme found in cat and beef liver (28,29). Current work is directed toward isolating a monoamine oxidase preparation from beef liver, following the method of Alles and van Heergaard (28). Briefly, the procedure consists of preparing a homogenate consisting of 4 parts water, 1 part liver, from which cellular debris is removed by centrifugation. The supernatant is dialyzed for 10 hours against distilled water. Upon adjusting the dialyzed preparation to pH 6, a precipitate forms containing monoamine oxidase activity. The precipitate is collected by centrifugation and resuspended in pH 7 phosphate buffer. Attempts to further purify this fraction by ammonium sulfate precipitation or other standard procedures have not been successful.

Some of the characteristics of this liver fraction have been investigated. It is capable of oxidizing ethylamine, propylamine, n-butylamine and phenylethylamine. Suspensions of this fraction stored at -20°C. have remained active for several weeks and it can be lyophilized and stored as a dry powder at -20°C. with little or no loss of activity.

The penetration of a 1 molar solution of n-butylamine through normal intact rat skin was determined using the monoamine oxidase preparation from beef liver to assay

the penetration rate. The results presented in Figure 11 indicate a steady-state oxygen uptake of approximately 300 µl O_2/hr., which in terms of the rate of amine penetrating the skin would correspond to 2.48 µmoles/cm.^{2}/hr. While this result is subject to confirmation in future experiments, it represents the highest penetration rate through normal rat skin of any compound tested so far except water.

Before launching into a full-scale investigation of amine penetration through skin, additional work will be required in order to obtain a satisfactory enzyme system. The current preparation consists of a rather nondescript precipitate which has resisted further purification. The major shortcoming of this preparation is its low specific activity, being on the order of 4-5 µl O_2/mg/hr. with n-butylamine as substrate.

An enzyme system satisfactory for studying the penetration of amines through skin should have at least a 5 to 10 fold higher specific activity than the present preparation. A more active preparation may be obtained by starting with isolated beef liver mitochondria, instead of working with a crude homogenate. It may be possible by rupturing the mitochondria to extract the enzyme in a soluble form from which a fraction of higher specific activity can be prepared.

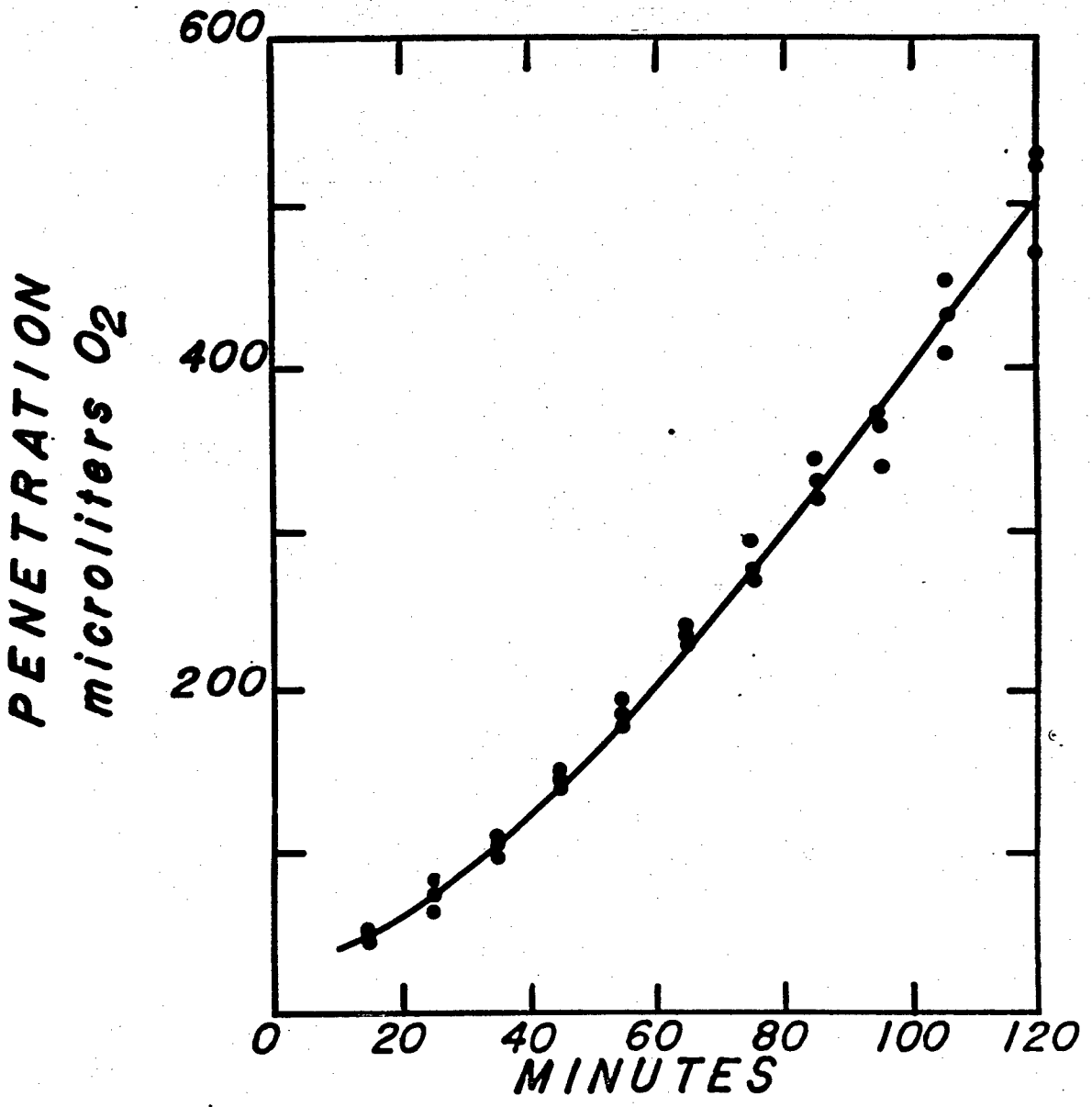


FIGURE 11. Penetration of n-Butylamine through Normal Rat Skin

VII. EPIDERMAL PROTEINS

A program to study the feasibility of preparing skin models has been initiated. As an initial step in this direction, work is in progress on the preparation of films of epidermal proteins and on subsequent investigation of the structural characteristics of these films.

It is generally conceded that most, if not all, of the barrier functions of the skin reside in the stratum corneum. This layer is a cellular tissue and little is known of its molecular structure. The region is largely proteinaceous, containing in addition about 8% lipid and small amounts of polysaccharide (30). The structural proteins of the epidermis have been divided into the keratins, those proteins solubilized only after the breaking of covalent bonds by reduction, oxidation or hydrolysis, and the pre-keratins, the proteins solubilized by reagents such as aqueous urea or lithium bromide which presumably disrupt only non-covalent linkages such as hydrogen bonds (31,32).

The proteins extractable from epidermis with aqueous urea have been studied extensively by Rudall (33) and by Carruthers, Woernley, Baumler and Kress (34). These workers used cow's nose epidermis, which is some 1.0 to

1.5 mm. thick, is practically hair-free, is low in lipids, and is readily available in large quantities. The epidermis was isolated and extracted with 6M urea and after dialysis to remove the urea, a non-fibrous, water-soluble protein fraction, having an isoelectric point of about pH 4.5, was isolated. Films of this protein had a β-type X-ray diffraction pattern. A fibrous protein, having an isoelectric point of about pH 5.5, was also isolated. This protein, epidermin, could be dried as a film which showed an α-type diffraction pattern when oriented by stretching in cold water. This type of X-ray diffraction pattern is characteristic of the stratum corneum and of the tonofibrils of the stratum mucosum (35). Both of these fractions have been shown to be complex mixtures by electrophoretic (34) and sedimentation studies (36). The major component of the urea extract was a mixture of very insoluble proteins having an isoelectric point near 6.3 which precipitated as a firm clot when the extract was dialyzed against water. Because of the insolubility of these proteins, this fraction has not been studied as thoroughly as the other two fractions. These three protein fractions will be designated simply as 4.5, 5.5 and 6.3.

More recently, Roe has used concentrated solutions of lithium bromide to extract a protein similar to epidermin,

the pH 5.5 protein of Rudall, from human epidermis (37). This protein was named tonofibrin because it was thought to be the structural protein of the tonofibrils. Tonofibrin, like epidermin, forms a fibrous precipitate near its isoelectric point of pH 5.5, and oriented films of this protein show an α-type X-ray diffraction pattern (38). Tonofibrin behaves as a single component on paper electrophoresis in borate buffer at pH 9.4. Other media that have been used to extract epidermal proteins include neutral phosphate and acetate buffers (30,39), borate buffer of pH 9.4 (40) and dilute sodium hydroxide solutions (41).

Initial attempts in our laboratory to prepare protein films suitable as skin models were directed at the use of the proteins obtained by lithium bromide extraction. This material was isolated from calf skin by an adaptation of the method of Roe (42). Calf epidermis was separated from "Zip" wax epilated skin with NH_4OH vapor and powdered by homogenizing with ethyl ether. The dried tissue was extracted at room temperature with 75% LiBr for 48 hrs. in sealed flasks with agitation. The mixture was clarified by centrifugation and the supernatant was dialyzed at room temperature against water at pH 7.0. The dialyzing fluid was agitated continuously and changed frequently until a negative test

for bromide was obtained with silver nitrate (about 48 hours). During this time, a white solid separated. The dialyzed extract was centrifuged at 2000 r.p.m. for 15 minutes. The precipitate was washed three times with water, three times with acetone and then air dried. The yield of protein was about 0.6% of the dry weight of the epidermis. Acidification of the supernatant to pH 4.5 gave an additional 1.5% yield of proteins.

It is planned to characterize the epidermal proteins by monolayer studies. Preliminary results have been obtained on an unpurified preparation of the proteins isolated by the above procedure. It was found that the crude dry proteins did not spread on the surface of acidic, neutral or 0.001N sodium hydroxide solutions. When the proteins were placed on the surface of 0.01N sodium hydroxide, a film formed which yielded typical pressure vs. area curves. A representative curve is shown in Figure 12. The area units are arbitrary as the protein concentration is unknown.

The proteins isolated by Roe's method and epidermin (the pH 5.5 protein isolated from urea extracts) are only minor constituents of epidermis. Between 1.5 and 2.5 g. of epidermis were obtained from one calf skin; this yielded from 30 to 50 mg. of epidermal protein after

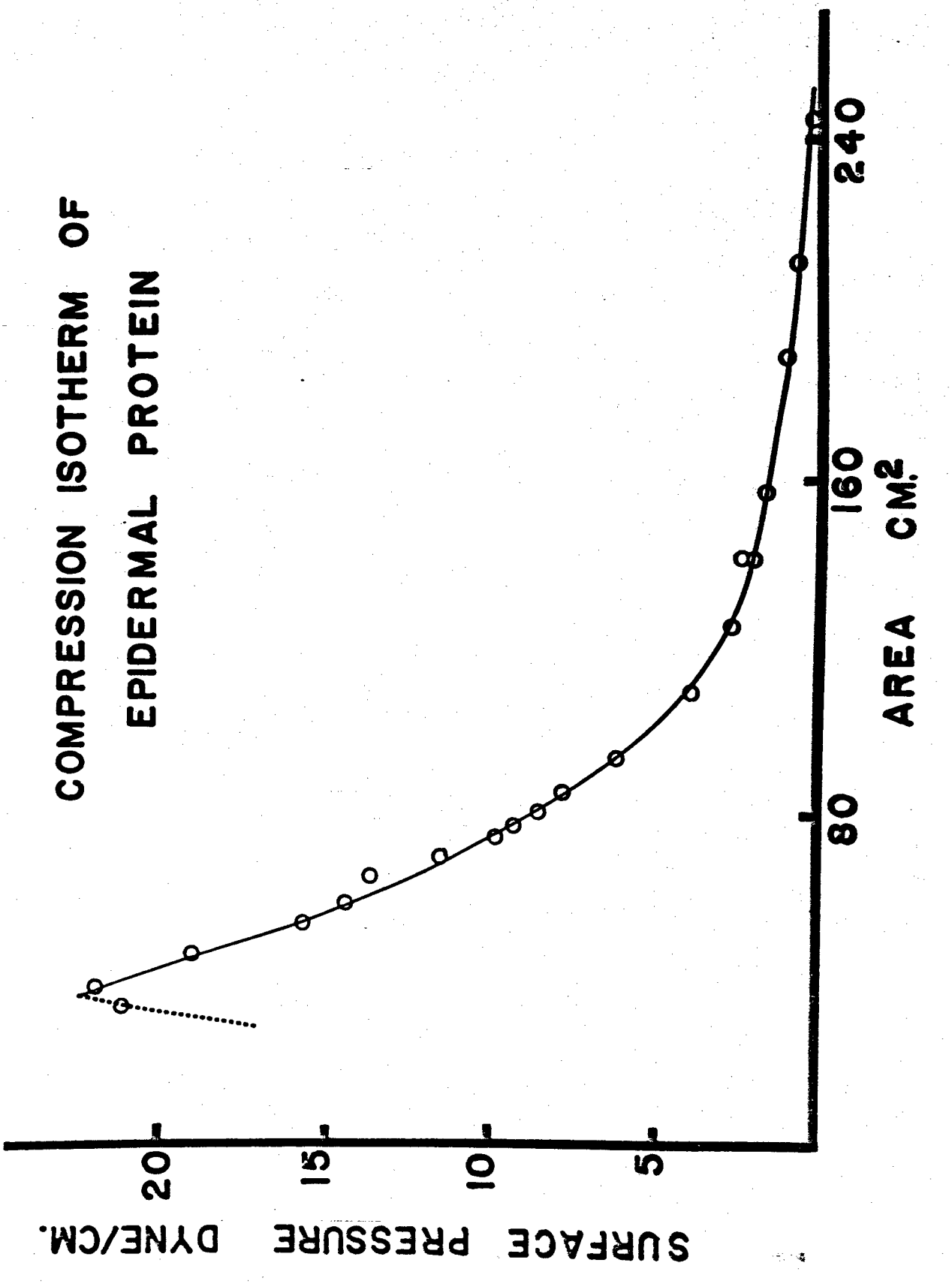

COMPRESSION ISOTHERM OF
EPIDERMAL PROTEIN
SURFACE PRESSURE DYNE/CM.
20
15
10
5
80
160
240
AREA CM.²

FIGURE 12

LiBr extraction. While such a yield may be satisfactory for characterizing the protein, it would be impractical as a source of proteins for skin models. Moreover, it has been found that both of these proteins are somewhat soluble in water. Attention is currently, therefore, being focused on the pH 6.3 protein isolated from beef snout by 6M urea. This protein is isolated by the following method.

Fresh beef snouts are obtained in lots of about 25 at the slaughter house and are immediately cooled by placing in crushed ice. All subsequent operations are carried out as close to 0° as possible. The snouts are trimmed and cut into 2 cm. wide strips. The strips are then cut parallel to the epidermal surface with a Castroviejo Keratotome set to cut to a depth of 1.0 mm. This procedure removed most of the epidermis just above the dermal junction. Only rarely did the samples contain dermal tissue. The epidermis, about 100 g., is homogenized at high speed with 500 ml. of 6M urea in a Waring Blendor for 4 minutes. During this time the mixture, initially at 2 to 4°C., warms to room temperature. The homogenate is cooled in an ice bath and placed in a refrigerator for 3 to 4 days. After the unextracted residue has been separated by centrifugation at 15,000 r.p.m. at 0°, a highly viscous solution is

obtained containing about 35 mg. of protein per ml. The extract is dialyzed for 24 hours against frequent changes of water. Five milliliters of a buffer containing 0.012 moles of K_2HPO_4 and 0.088 moles of NaH_2PO_4 per liter is added to each gallon of distilled water used as dialyzing fluid to keep the pH above 6.3 and prevent precipitation of the pH 5.5 protein. (In some of the earlier experiments a pH 6.8 phosphate buffer was employed.) The mixture is briefly homogenized, to break up the firm clot that is formed on dialysis, and is centrifuged at 15,000 r.p.m. for 10 minutes at 0°. The precipitated pH 6.3 protein is suspended in water, recentrifuged and stored moist until use.

The pH 6.3 protein is soluble in dilute alkalies and acids. Films have been cast from such solutions. Work is currently in progress to develop a procedure that will provide films with reproducible physical characteristics.

VIII. STUDY OF DINITROCHLOROBENZENE (DNCB) PENETRATION OF SKIN EMPLOYING THE FLUORESCENT ANTIBODY TECHNIQUE

In the present study the fluorescent antibody technique of Coons (43) is being employed in an effort to visualize degree and route of penetration of a sensitizing chemical through skin. The approach being used is based on the rationale that:

1) A sensitizing chemical, such as DNCB, has the capacity of penetrating epidermis.

2) DNCB, by virtue of its slow rate of combination with protein under *in vivo* conditions, reacts with tissue protein all along its route of penetration through epidermis.

3) Antibody which will react with the *in vivo* DNCB-tissue protein complex can be induced in animals by immunization with a dinitrophenylated protein.

4) The (anti-DNCB-tissue protein) antibody, after being fluoresceinated, will enable visualization of the path of penetration of the sensitizing chemical.

Initial phases of this study are described herein. Preliminary steps have been discussed previously (7).

Immunochemical Studies

Dinitrophenyl-bovine plasma albumin (DNP-BPA) was synthesized from BPA (Armour) and dinitrofluorobenzene by the method of Sanger (44), modified so as to retain the water solubility of the conjugate. The DNP-BPA was suspended in complete Freund's adjuvant (Difco Co.), and immunization of guinea pigs carried out following the procedure of Proom (45). By this means, active antiserum has been obtained. Efforts to obtain antiserum with DNCB alone were unsuccessful.

The results of specificity testing of the antiserum against various materials by Ramon titration (46) are given in Table XIII.

Table XIII

RAMON TITRATION OF SERUM OF GUINEA PIGS IMMUNIZED WITH DINITROPHENYL-BOVINE PLASMA ALBUMIN

Antiserum: In all cases from DNP-BPA-immunized guinea pig.

Material Tested	Flocculation Reaction
DNP-BPA	++++
BPA	+++
Human albumin (HA)	-
Beta-lactoglobulin (BLG)	-
DNP-HA	+
DNP-BLG	+

It may be inferred from the above data that the antiserum possesses antibody fractions with activity 1) against the whole antigen, i.e., DNP-BPA, 2) against the protein moiety of the antigen, i.e., BPA, and 3) against the DNP-grouping alone. The activity against the DNP-grouping is shown by the positive flocculation obtained when this grouping is introduced into two proteins (HA and BLG) which before dinitrophenylation gave no flocculation reaction when tested with the antiserum.

The globulin fraction of the antiserum was isolated and conjugated with fluorescein isothiocyanate (Nutritional Biochemicals Corp.) and prepared for tissue staining essentially as described by Cohen et al. (47), except that Sephadex G-25 was used for removal of free fluorescein derivatives after conjugation, and rat liver rather than the more fibrous pork liver was used for the powder adsorption to remove non-specific staining.

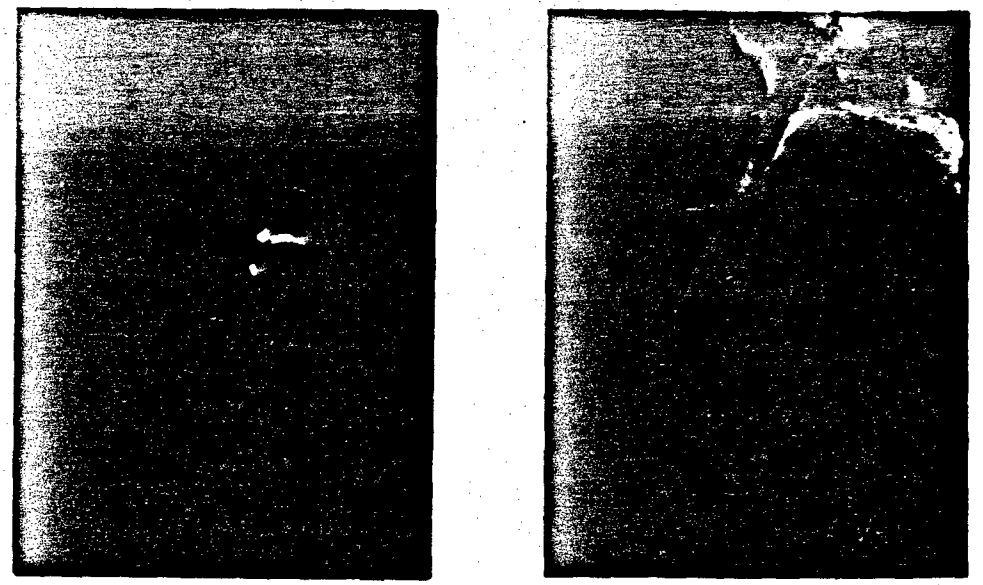
Cytologic Study

Guinea pigs were treated topically on one dorso-lateral surface with 0.05 ml. of a 0.5% solution of DNCB in 95% ethanol (EtOH) on two successive days, and sacrificed on the third day. The other dorso-lateral surface was treated with ethanol alone. Tissue from the DNCB/EtOH- and EtOH-treated areas was snap-frozen in a dry ice-isopentane

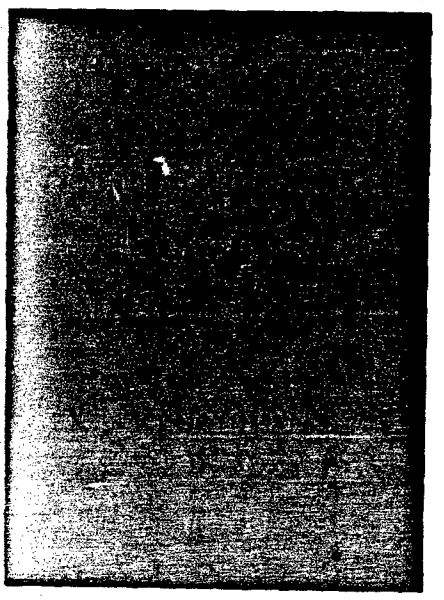
mixture. Cryostat sections, 5-6 microns in thickness, were prepared, and, after fixation in acetone, stained with one of the following reagents: 1) fluoresceinated gamma globulin from active antiserum of a DNP-BPA-immunized guinea pig (Ab-F); 2) non-fluoresceinated whole antiserum from DNP-BPA-immunized guinea pigs (Ab), followed by washing and treatment with Ab-F.

In this preliminary study, normal and EtOH-treated sections were tested only with reagent "1" (Ab-F). Reagent "2" (Ab + Ab-F) was employed to define the immunologic specificity of any fluorescence observed by ultraviolet microscopy when DNCB-treated sections were tested with reagent "1" (Ab-F).

A section from an EtOH-treated control area (Figure 13a) of a guinea pig, when tested with Ab-F, showed a thin band of greenish fluorescence only in the region of the stratum corneum conjunctum. A section from a DNCB-treated area (Figure 13b), when tested with Ab-F, showed bright greenish-yellow fluorescence in the stratum corneum and hair shaft. Fluorescence was also evident in the entire epidermis including the pilosebaceous duct. A serial section from the same DNCB-treated area, when tested by Ab + Ab-F, showed prominent fluorescence only in the stratum corneum (Figure 13c).



a b



c

FIGURE 13 a. Section of Untreated Guinea Pig Skin after Exposure to Fluoresceinated Gamma Globulin Fraction of Anti-(DNP-BPA) Guinea Pig Serum (Ab-F)

13 b. Section of DNCB-Treated Guinea Pig Skin after Exposure to Ab-F

13 c. Section of DNCB-Treated Guinea Pig Skin Treated with Ab-F after Prior Exposure to Blocking Antibody

Rappaport (48) studied the skin of humans sensitized to egg albumin. Human skin biopsies from sites challenged by egg albumin were tested with human (anti-egg albumin) reaginic serum, as well as with antibody from rabbits immunized with egg albumin. The antibody preparations were tagged with fluorescein isothiocyanate. He found by this method of testing that in antigen-challenged skin, all cells of epithelial origin showed specific fluorescence, including the Malpighian layer, hair follicles, and sebaceous and sweat glands. It was observed by Rappaport, however, that the stratum corneum stained intensely and non-specifically in all skin preparations studied including controls.

While we have studied the route of allergen penetration through epidermis, Rappaport has concentrated on allergen distribution within the challenged skin. The results of our preliminary observations, however, appear to agree with Rappaport's findings, regarding general distribution of fluorescence throughout the epidermis of the allergen-treated skin.

IX. SUMMARY

Alterations in the skin of rats maintained on a fat-free (EFA-deficient) diet were studied by biological, chemical and physical techniques. Skin from EFA-deficient rats presented a picture of parakeratosis, acanthosis and hypertrophy of the sebaceous glands. Protein sulfhydryl groups were far more prominent and extensive throughout the epidermis (particularly in the stratum corneum) than observed in normal skin. Total epidermal sulfhydryl gave an average value almost double that of the controls, while epidermal disulfide was not significantly changed. The water diffusion rate and electrical conductivity of skin rose sharply after six weeks of feeding the EFA-deficient diet. Also, EFA-deficient skin was more permeable to organic acids, amino acids, glucose and urea than normal skin.

The skin lipids of normal and EFA-deficient rats are being analyzed using thin layer and gas-liquid chromatography. Preliminary results indicate differences between skin lipids of a control rat and an EFA-deficient rat, both in the lipid profile as obtained by thin layer chromatography and in fatty acid composition.

In contrast to the increased permeability of skin from rats deficient in EFA, the permeability of skin from rats

on diets deficient in vitamins A, B_6 and C was not altered. These skins did show histological changes which apparently did not affect the barrier region. Skin permeability did not change in cases of magnesium deficiency or when low or high protein diets were fed to rats.

The permeability of skin of animals treated with various solvents was studied. Ethanol treatment had no effect, while treatment with olive oil and with petroleum ether did cause a small increase in the permeability of skin to both water and ions. After acetone treatment, however, a great increase in the permeability of skin to water, ions, and urea was seen. Histologically, minor skin alterations were noted only after petroleum ether and acetone treatments.

Preliminary experiments indicate that n-butylamine penetrates normal rat skin readily, as measured in the skin penetration vessel employing monoamine oxidase to detect the amine after passage through the mounted skin.

Two methods for isolating structural proteins from epidermis, one involving aqueous urea and the other aqueous lithium bromide as extractant, were compared in regard to their suitability for preparing protein skin models. Both methods provided protein preparations from which cohesive films could be cast. The urea extraction method is currently being used because it gives better yields of epidermal protein. A compression isotherm of epidermal

protein isolated by the lithium bromide extraction was recorded.

The penetration of dinitrochlorobenzene (DNCB) into skin was studied by a fluorescent antibody technique, employing antibody with activity for the dinitrophenyl grouping. In preliminary studies, fluorescence was observed throughout the epidermis of non-sensitized guinea pigs treated with DNCB. Particularly high intensity was seen in the pilosebaceous duct. Control sections showed prominent fluorescence (non-specific) only in the stratum corneum.

X. FUTURE ACTION

Clues to the nature of the barrier are found in studies on skin of EFA-deficient rats. Morphologically EFA-deficient skin presents a picture of accelerated growth evident in the sebaceous glands and in all layers of the epidermis. One outstanding feature of hypertrophied epidermis is the high concentration of sulfhydryl groups in all layers indicative of an inability to form disulfide cross linking (impaired keratinization process).

As observed also by Sinclair and other investigators, the deficient skin lost water readily. Also, the electrical conductivity of skin increased in the EFA-deficiency state and this permeability to ions appeared to correlate closely with the increased loss of water.

These observations would indicate that in EFA-deficient rats, the epidermal barrier has become more porous than in normal skin. The easy penetration of chemical agents like urea and organic acids into EFA-deficient skin fits in well with this concept. However, it is interesting to note that other simple compounds like glucose and glutamic acid pass through skin with difficulty, even in the EFA-deficiency state. This would indicate that the skin still provides a formidable barrier to some compounds even when deficient in EFA. Glucose was ob-

served to show only slow penetration through the skin even with the epidermis removed. Studies are planned to study the relative penetration rates of other sugars including those without reducing groups.

Studies on the nature of the barrier regions of normal and EFA-deficient skin are being extended to include characterization of extracted epidermal proteins by zonal electrophoresis and amino acid analyses. The chemical and histochemical evidence of impaired sulfur metabolism in skin of EFA-deficient rats is strong indication that the protein structure in the corneum layer is a functional part of the barrier region.

The finding that skin fat composition is also considerably altered from that of normal skin has focused our attention on the significance of the different lipid materials in contributing to the "barrier" make-up. Preliminary findings employing gas liquid and thin layer chromatography have been very encouraging in establishing differences in skin lipid samples. Further studies have been initiated which will provide results on detailed lipid composition of the epidermis under different stress states.

Another finding of significance was the action of acetonewhich apparently had a greater effect on increasing

skin permeability than other organic solvents tested. Evidence is presented to indicate that after one treatment, acetone, in addition to the expected defatting action, has a deep seated effect on the epidermis as manifested by changes in the basal layer cells. Other polar solvents having both lipid and water compatibility such as dimethyl formamide, carbitol, pentaerythritol, dimethyl sulfoxide, dimethyl phthalate and 2-butoxyethanol will be investigated relative to their effects on skin permeability.

The following enzymes in normal and EFA-deficient skins will be histochemically compared:

Alkaline phosphatase
Acid phosphatase
Succinic acid dehydrogenase
Monoamine oxidase
Cytochrome oxidase
β-glucuronidase
α-naphthol esterase
Choline esterase
Amino peptidase

Because of unfortunate delays no report can be made at this time on skin structures examined electron microscopically. Presently, the Philips Electron Microscope (Model EM 100B/15) has been assembled and is in working

order. Skin specimens discussed in this report will be examined under the electron microscope for ultramicroscopic detail which would delineate structural differences between normal skin and skin made permeable by a variety of treatments.

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